A Defined Medium for *Haemophilus influenzae* and *Haemophilus parainfluenzae*

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SUMMARY

A defined medium is described for the growth of *Haemophilus influenzae* and *H. parainfluenzae* which can be used either as a liquid medium or solidified with agar. The purine and pyrimidine, vitamin, amino acid and mineral salt requirements for three strains have been analysed.

INTRODUCTION

Although a chemically defined medium has been described for *Haemophilus parainfluenzae* (Herbst & Snell, 1949; Herbst & Glinos, 1955), no such medium has been available for *H. influenzae*. This has retarded progress in the study of the genetics of transformable haemophilus strains since biochemical markers could not be studied. It was the possible application in genetic studies that prompted the search for a defined medium for *H. influenzae*. Since the start of this investigation Talmadge & Herriott (1960) described a modification of the tissue culture medium M-199 (Morgan, Morton & Parker, 1950) which allowed the growth of *H. influenzae*. It was felt, nevertheless, that further study was needed and that perhaps a simpler defined medium might be devised.

METHODS

Organisms

Haemophilus influenzae strain Rd of Alexander & Leidy (1958), a rough mutant from a wild Pittman type d, and of known transformability; *H. influenzae* strain RdTr, a strain derived from strain Rd by Dr P. Schaeffer, resistant to streptomycin, erythromycin and novobiocin; and *H. parainfluenzae* strain FID, isolated from a human throat by Dr H. E. Alexander; these will be referred to in the text as strains Rd, RdTr and FID. All strains were maintained on slopes of chocolate agar in cotton-wool plugged test tubes kept at 37°.

Stock culture medium

Tryptone (Difco) was dissolved in glass distilled water at 20 g. together with NaCl at 10 g./l. The pH was brought to about 8 with HCl and activated charcoal added to give a 0.2% (w/v) suspension. The mixture was allowed to stand with occasional shaking for 15 min. and then filtered. The filtrate was adjusted to pH 7.5 and agar (Davis) added to give a 2% (w/v) solution. The medium was stored at this stage. When required, oxalated horse blood was added to give a 5% (v/v) mixture to the molten medium cooled to about 80°, and the blood allowed to turn to a chocolate colour, heated further in a water bath when necessary. The medium was then dispensed as slopes or plates.

Solutions used in the development of the defined medium

Haematin solution. A stock solution of haematin (Roche Products Ltd.) was prepared as follows: 50 mg. haematin were mixed with 12.6 ml. 0.5 M- Na_2HPO_4 , heated to dissolve, and 86 ml. glass distilled water + 1.6 ml. M- KH_2PO_4 added. The solution was sterilized by autoclaving at 115° for 10–15 min. One ml. of the stock solution was used in 21 ml. of medium, i.e. a final concentration of 2.4 mg. haematin/100 ml. medium.

Coenzyme 1 solution. Diphosphopyridine nucleotide (Sigma Chemical Co.) was dissolved in glass distilled water to 1 mg./ml., 0.06 ml. of this solution was used in 21 ml. medium, i.e., a final concentration of 0.3 mg. DPN/100 ml.

Glucose-salts solution 1. The solution consisted of 5.0 g. glucose; 17.5 g. K_2HPO_4 ; 5.0 g. KH_2PO_4 ; 1.056 g. Na_3 citrate. $5\text{H}_2\text{O}$; 0.25 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2.5 g. $(\text{NH}_4)_2\text{SO}_4$ in 100 ml. glass distilled water. The mixture was sterilized by autoclaving at 115° for 10 min., and 0.8 ml./21 ml. medium was used.

Glucose-salts solution 2. The solution consisted of 20 g. glucose; 17.5 g. K_2HPO_4 ; 5.0 g. KH_2PO_4 ; 0.105 g. Na acetate; 0.25 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g. $\text{Ca}(\text{NO}_3)_2$; 0.006 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.625 g. NaHCO_3 in 100 ml. glass distilled water. The mixture was sterilized by autoclaving at 115° for 10 min.; 0.4 ml. was used per 21 ml. medium.

Yeast extract solution. Yeast extract (Difco) was dissolved in glass distilled water to make a 10% (w/v) solution, and sterilized by Seitz filtration.

Zeo-carb treated yeast extract. Yeast extract solution (10% (w/v)) was treated with Zeo-carb 225/H in the proportion of 1 g. resin/3 ml. solution for 20–25 min. with occasional shaking, the mixture then centrifuged, and the supernatant fluid removed and sterilized by Seitz filtration.

Yeast extract ash solution. Yeast extract was ignited repeatedly to constant weight in a platinum dish. The ash was dissolved in concentrated nitric acid, glass distilled water added and the solution adjusted to pH 6.5. Further distilled water was added to give a concentration equivalent to 10% (w/v) of the initial weight of yeast extract before ashing. The solution was sterilized by autoclaving at 115° for 10 min.; 0.5 ml. of this solution was used in 21 ml. medium.

Agar. New Zealand agar (Davis) at 3% (w/v) in glass distilled water was sterilized by autoclaving. The molten agar was mixed with the other medium constituents in 25 ml. screw-capped bottles and then poured in plates, the final agar concentration being 1% (w/v).

Evaluation of medium

Suspensions of the organisms were made in normal saline and a loopful spread over a segment of a plate of medium. After incubation at 37° for 24 hr. and 40 hr. a visual assessment was made of the density and homogeneity of growth and of colony size. Assessment of growth in liquid cultures was made by a visual comparison of the turbidities.

RESULTS

It was decided to start from a non-defined medium which supported growth and which might lend itself to separation into its components. The basal solution contained haematin and DPN as indicated above, with 200 mg. crystalline bovine

albumin (Armour Pharmaceutical Co. Ltd.) per 100 ml. medium, and either glucose-salts solution 1 or glucose-salts solution 2. To this mixture were added the other constituents to be studied, followed by the agar. The albumin requirement will be discussed later.

Yeast extract

Strain FID grew well on basal solution + glucose-salts solution 1 containing yeast extract at a final concentration of 0.25 % (w/v) and above, and still gave small colonies with the yeast extract down to 0.05 % (w/v). Strain Rd also grew but not as well, requiring 40 hr. to give colonies. A somewhat more luxuriant growth was obtained with strain FID on the addition of the 19 amino acids shown in column (a) of Table 1, and the growth developed brown pigmentation. Strain Rd also grew better in the presence of the amino acids, forming dense white colonies with a more transparent rim, and causing a white precipitate to appear in the medium. These colonies were found to have a decreased requirement for haematin and were designated Rd1; the properties of this variant will be described elsewhere.

Investigation of yeast extract constituents

Diffusibility. When a sterile dialysis sheet (Visking) was placed over the medium containing double strength yeast extract, the sheet covered with an equal volume of basal solution + glucose-salts solution 1 + amino acids, and the surface inoculated, strain Rd did not grow although strain FID gave pigmented colonies. This suggested that the activity given by the yeast extract which allowed growth of strain Rd was not dialysable. This point was not investigated further.

Effect of Zeo-carb treatment. Addition of yeast extract treated once with Zeo-carb to the basal solution + glucose-salts solution 1 at a final concentration equivalent to 0.25 % (w/v) of untreated yeast extract gave decreased growth of strain FID even in the absence of added amino acids; strain RdTr behaved similarly but only when the concentration of the treated yeast extract was doubled. Strain Rd did not grow even in the presence of added amino acids. After the once-treated yeast extract had been autoclaved at 115° for 10 min., it lost the ability to support strain RdTr, and the growth of strain FID was further diminished. The addition of amino acids stimulated strain FID but had no effect on strain RdTr.

Treatment of the yeast extract three times with Zeo-carb followed by Seitz filtration or autoclaving resulted in a still further diminished ability to support strains FID and RdTr.

In summary, treatment of the yeast extract three times with Zeo-carb followed by autoclaving still permitted some growth of strains RdTr and FID in the absence of added amino acids. Strain Rd did not grow after such treatments of the extract. These findings suggested that, given a medium containing a full complement of amino acids and vitamins, the additional essential constituents provided by untreated yeast extract and removed by resin treatment might include purines, pyrimidines and metal ions.

Replacement of yeast extract

The ability of medium containing treated yeast extract + basal solution + glucose-salts solution to support full growth of all three strains was restored by adding to it a solution of the ten vitamins shown in column (b) of Table 1, together with the nineteen amino acids and a solution of ash prepared from yeast extract. Later it was found that the Zeo-carb treated yeast extract itself could be replaced by a solution of purines and pyrimidines. Finally, the ash solution could be replaced by a solution of zinc sulphate.

Table 1. *Mixtures of amino acids and of vitamins used as additions to the basal medium*

(a)		(b)	
Amino acid	mg./l.	Vitamin	mg./l.
Glycine	45.7	<i>p</i> -Aminobenzoic acid	8.0
DL-Alanine	45.7	Thiamine	8.0
DL-Valine	187.1	Biotin	8.0
L-Leucine	187.1	Ca pantothenate	8.0
DL-Isoleucine	76.2	Choline chloride	8.0
DL-Serine	187.1	Folic acid	0.8
DL-Threonine	68.55	Inositol	8.0
DL-Aspartic acid	187.1	Nicotinic acid	8.0
L-Glutamic acid	228.5	Pyridoxine HCl	8.0
L-Arginine HCl	45.7	Riboflavin	0.8
L-Lysine (HCl) ₂	187.1		
L-Cysteine HCl	118.9		
DL-Methionine	68.55		
Glutathione	59.5		
DL- β -Phenylalanine	68.55		
L-Tyrosine	76.2		
L-Tryptophan	22.9		
L-Histidine HCl	56.7		
L-Proline	187.1		

Purine and pyrimidine requirements

The purine and pyrimidine requirements were investigated by using the basal solution + glucose-salts solution 2 + amino acids and vitamins indicated above, together with glutamine + putrescine + yeast extract ash. The purines used were adenine, guanine and hypoxanthine, and the pyrimidines thymine, cytosine and uracil. The results obtained are summarized in Table 2, from which it can be seen that the requirements of all three strains were met by the inclusion of only adenine, guanine and uracil. Strain Rd required adenine and uracil, whilst strain FID required guanine, although uracil, and to a lesser extent, adenine, stimulated growth. A detailed analysis of the requirements of strain RdTr was not made, but it grew well on the mixture containing adenine, guanine and uracil. Neither thymine, cytosine nor hypoxanthine was required by any of the three strains.

Table 2. *Purine and pyrimidine requirements of three Haemophilus strains*

Strain	Essential	Stimulatory	Not required
Rd	Adenine, uracil	—	Thymine, cytosine, hypoxanthine, guanine
FID	Guanine	Uracil, adenine	Thymine, cytosine, hypoxanthine
RdTr	Grows well with adenine + guanine + uracil		

Vitamin requirements

Of the ten vitamins listed in column (b) of Table 1, *p*-aminobenzoic acid, biotin, choline chloride, folic acid, inositol, nicotinic acid and riboflavin were not required by any of the strains. Table 3 gives the results for the remaining vitamins, and also for glutamine and putrescine. The latter substance was used because of the work of Herbst & Snell (1949) in which putrescine was described as a growth factor for *Haemophilus parainfluenzae*. It can be seen from Table 3 that no added vitamin appeared to be essential for strain Rd, although calcium pantothenate and thiamine were stimulatory, the former being replaceable by pyridoxine. Strains RdTr and FID both required pyridoxine, and were stimulated by pantothenate, although this could be replaced by thiamine for strain FID. Putrescine stimulated all three strains and glutamine was stimulatory for strain RdTr. It was observed that, in the presence of added biotin and folic acid, neither of which was required, strain RdTr then showed a requirement for thiamine. The inclusion of pyridoxine, thiamine, calcium pantothenate, putrescine and glutamine therefore met the vitamin requirement of all three strains.

Table 3. *Vitamin requirements of three Haemophilus strains*

Strain	Essential	Stimulatory	Not required
Rd	—	Ca pantothenate, putrescine, (pyridoxine in absence of Ca-pantothenate)	Biotin, folic acid, thiamine, glutamine, pyridoxine
RdTr	Pyridoxine (thiamine in presence of biotin and folic acid)	Ca pantothenate, glutamine, putrescine	Biotin, folic acid
FID	Pyridoxine	Ca pantothenate, putrescine (thiamine in absence of Ca-pantothenate)	Biotin, folic acid, thiamine, glutamine

Mineral salts requirement

The salts finally used are shown in Table 5.

ZnSO₄. It was found that the yeast extract ash could be replaced by a suitable concentration of ZnSO₄. The element Zn is a known constituent of some enzyme systems. At a concentration of 8.42 mg. ZnSO₄.7H₂O/100 ml. medium (0.0001 M) the growth promoting effect was at least as good as that of the yeast extract ash at the concentration previously stated, but at lower concentrations of the zinc salt there was a rapid diminution of growth. This concentration of zinc represents approximately 1 part/2500. Zinc was spectroscopically undetected in the yeast extract ash but this concentration is below the limit of detection by this method in a sodium and potassium matrix.

Na acetate. This compound had no effect on strains Rd, RdTr or FID. It was retained in the complete medium, however, because it was stimulatory to the growth of the variant Rd1 referred to above, which was investigated simultaneously during the development of this medium.

NaHCO₃. This was stimulatory to all three strains.

FeSO₄. This was somewhat stimulatory to all three strains at 0.114 mg. FeSO₄.7H₂O/100 ml. medium (0.000004 M), but was toxic at higher concentrations.

Phosphates. At concentrations lower than 330 mg. K_2HPO_4 /100 ml. medium (0.019 M) and 94 mg. KH_2PO_4 /100 ml. medium (0.007 M) there was diminished growth.

CaCl₂. When the medium contained crystalline bovine albumin, $CaCl_2$ was not required to be added, but when the albumin was replaced by polyvinyl alcohol, then $CaCl_2$ at a concentration of 6.75 mg./100 ml. medium (0.0006 M) was stimulatory.

MnCl₂. This was not found to be necessary.

In general strains FID and RdTr were more sensitive to higher concentrations of salts than was strain Rd; strain RdTr was the most sensitive.

Amino acid requirements

Of the nineteen amino acids listed in column (a) Table 1, glycine, alanine, serine, threonine, methionine, proline and lysine could be omitted without decreased growth. Glutathione and cysteine were interchangeable, but better growth was given with glutathione. Table 4 gives a summary of the amino acid requirements for each strain. It will be noted that strain Rd did not appear to have an essential requirement for any one amino acid, i.e. the omission of any one of the amino acids from the minimal mixture did not abolish growth.

Table 4. *Amino acid requirements of three Haemophilus strains*

Strain	Essential*	Stimulatory	Not required
Rd	—	Tryptophan, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, isoleucine, phenylalanine, valine	Lysine, glutathione or cysteine, methionine, alanine, glycine, proline, threonine, serine
FID	Isoleucine, valine	Tryptophan, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, phenylalanine	As for strain Rd
RdTr	Isoleucine, leucine, tyrosine	Tryptophan, cysteine or glutathione, aspartic acid, valine (histidine, arginine, and phenylalanine not tested with strain RdTr)	Glutamic acid and as for strain Rd

* Where omission of any one amino acid from the minimum mixture stopped growth.

Albumin

It had been observed that the strains used, *Haemophilus parainfluenzae* in particular, did not grow well in broth media unless albumin was also added, and it seemed likely that the albumin was acting as a protective colloid. Consequently, crystalline bovine albumin at a final concentration of 0.19 % (w/v) had been added to the defined medium throughout its development. It was found, however, that this concentration was having an inhibitory effect; by decreasing the concentration a peak of maximum growth was obtained at a final concentration of 0.019 % (w/v), with a sharp decrease in growth at lower concentrations and a more gradual decrease at higher concentrations; the curve obtained is shown in Fig. 1. Such a result would be expected if the albumin indeed acted as an adsorbent, and an alternative was sought to take its place. Polyvinyl alcohol was tried, with some success. The curve obtained by plotting concentrations of polyvinyl alcohol against growth is also shown in Fig. 1, and it can be seen that it has the same form as that for albumin. The slope

of the peak on the high concentration side is steeper and the growth was somewhat less than with albumin. However, at 64 mg. polyvinyl alcohol/100 ml. medium, the inclusion of CaCl_2 at 6.7 mg./100 ml. medium gave growth comparable to that on albumin.

The medium containing either albumin or polyvinyl alcohol proved very satisfactory for several weeks, but on repeating the experiment growth suddenly became poor and even failed completely. It is known that fatty acids may cling even to well-washed glassware. For this reason, the effect of adding sodium oleate was investigated. It was found that growth could be restored in this way. In the presence of albumin, good growth was obtained with 1.92 mg. sodium oleate/100 ml. medium or

Fig. 1. Effect of crystalline bovine albumin (solid line and open points) and polyvinyl alcohol (broken line and solid points) on the growth of *Haemophilus* strains—Rd (O, ●), RdTr (Δ, ▲), and FID (□, ■).

in the presence of polyvinyl alcohol with 0.96 mg./100 ml. medium. Strain Rd was affected only slightly by increase of oleate up to 25-fold with albumin, or to 10-fold with polyvinyl alcohol, but strains FID and RdTr were more sensitive. The latter strains were also more sensitive than strain Rd to lower concentrations of oleate. Oleate in fact completely replaced albumin or polyvinyl alcohol, best growth being obtained at a concentration of 0.48 mg. oleate/100 ml. medium.

Agar

Agar was added to give a final concentration of 1 % (w/v). Unwashed New Zealand (Davis) agar gave the same results when compared with New Zealand agar which had been washed 48 hr. in distilled water, and with Noble (Difco) agar.

The medium finally evolved, therefore, had the composition given in Table 5. The medium was prepared by mixing appropriate volumes of several solutions containing either mixtures of various substances or single components. The table shows the pH of the stock solutions, and whenever possible these were adjusted to about pH 7. Where no pH is indicated, no adjustment was made.

Viable counts (Miles & Misra, 1958) were carried out with suspensions of each

strain, comparing the counts obtained on the defined solid medium containing albumin or polyvinyl alcohol with those obtained on chocolate Tryptone agar. The counts obtained were similar, and showed that the defined media were equal to the chocolate agar in giving growth from inocula of 1 to 10 organisms. Such growth could not consist of selected mutants only. The colonies on the defined medium were somewhat smaller than those obtained on the chocolate tryptone agar and needed incubation for 36 hr. before counting.

Table 5. *Composition of chemically defined medium for certain strains of Haemophilus influenzae and H. parainfluenzae*

Except where indicated, constituents were sterilized by autoclaving at 115° for 10–15 min.

Substance	mg./l.	pH of stock solution	Substance	mg./l.	pH of stock solution
DL-Valine	137.1	6.9	Glucose	3810.0	
L-Leucine	137.1	6.9			
DL-Isoleucine	76.2	6.9	K ₂ HPO ₄	3300.0	7.6
DL-Aspartic acid	137.1	6.9	KH ₂ PO ₄	940.0	
L-Glutamic acid	223.5	6.9	ZnSO ₄ .7H ₂ O	34.2	
L-Arginine HCl	45.7	6.9	Na acetate	41.9	
DL-β-Phenylalanine	68.55	6.9	NaHCO ₃	225.0	
L-Tyrosine*	76.2	6.9	MgSO ₄ .7H ₂ O	180.0	
L-Tryptophan*	22.9	6.9	FeSO ₄ .7H ₂ O*	1.14	
L-Histidine HCl	56.7	6.9	NaCl	8000.0	
L-Cysteine HCl*	118.9				
or					
Glutathione*	59.5		Na oleate	4.8	
Adenine	9.5	6.5	Agar	10,000	
Guanine	0.95	2.0			
Uracil	0.95	6.5			
Pyridoxine HCl*	8.0	7.1			
Thiamine*	8.0	7.1			
Ca pantothenate*	8.0	7.1			
Glutamine*	300.0				
Putrescine*	2.98				
Haematin	23.8				
Coenzyme I*	2.9				

*Sterilized by Seitz filtration.

The defined medium without agar and with albumin allowed growth of all three strains, but only strains Rd and RdTr grew in liquid medium containing polyvinyl alcohol (but no oleate). This suggests that while crystalline bovine albumin or polyvinyl alcohol + CaCl₂ are satisfactory alternatives for an agar solidified medium only albumin should be used for liquid medium. The addition of oleate to the albumin-containing medium enhanced growth, particularly that of strains Rd and FID. Oleate without albumin gave somewhat less growth than with albumin.

Five other strains of *Haemophilus* obtained from the National Collection of Type Cultures (NCTC) were tested for growth on the solid medium. *Haemophilus influenzae* NCTC 4560 and *H. parainfluenzae* NCTC 4101 grew on both the albumin-containing medium and the polyvinyl alcohol-containing medium, whilst *H. canis* NCTC 1659 grew on the albumin-containing medium only. *H. aegyptius* NCTC 8502 and *H. haemolyticus* NCTC 8479 did not grow on either medium. The use of oleate, however

(with or without albumin), allowed growth of the *H. haemolyticus* and also of two isolates of *H. influenzae* recently obtained, one from an eye swab and the other from a specimen of sputum. The *H. aegyptius* strain was not tried on the oleate-containing medium as it was not available at the time of those experiments.

DISCUSSION

These results show that the nutritional difference between *Haemophilus influenzae* and *H. parainfluenzae* is not simply that the former is dependent on added haematin, since differences also occur between the two species in their amino acid, vitamin, purine and pyrimidine requirements, and in their sensitivities to the action of protective colloids. The dependence of *H. influenzae* on haematin, taken together with other cultural characteristics, gives the impression that this species is likely to be more exacting in its nutritional requirements than *H. parainfluenzae*. However, the results were contrary to such a concept, since the growth of strain Rd was not dependent on the presence of any one vitamin or amino acid (in the presence of other vitamin and amino acids), whereas that of strain FID was dependent on the presence of pyridoxine, isoleucine and valine. It is tempting to suggest that the need of strain RdTr for isoleucine, leucine and tyrosine is connected with the resistance of this organism to streptomycin, erythromycin and novobiocin.

The use of crystalline bovine albumin in a defined medium may be objected to. It seems probable that the albumin takes no part as a nutrient and acts only as an adsorbent of growth inhibitors and a substitute for it was sought. It was thought that charcoal would be too active, and indeed Brumfitt (1959) found that *Haemophilus influenzae* was inhibited by 0.5 % (w/v) charcoal although *Bordetella pertussis* grew better in its presence. Starch has been used by several workers (Hornibrook, 1939, 1940; Wilson, 1945; Farrell & Taylor, 1945; Cohen & Wheeler, 1946; Verwey, Thiele, Sage & Schuchardt, 1949) but it was thought that this might hydrolyse to give sugars which would interfere with the use of the medium in a study of the sugar reactions of the group. Talmadge & Herriott (1960) included polyvinyl alcohol in their modified M-199 medium, and it seemed probable that it was used as a detoxifying agent. As the present results showed, polyvinyl alcohol allowed growth comparable to that obtained with albumin (in the presence of low concentrations of Ca ion), but the strains tested were more sensitive to small changes in the concentration of polyvinyl alcohol than they were to similar changes of albumin.

The ability of oleate to replace albumin and polyvinyl alcohol would suggest that oleate also plays a physical rather than a metabolic role. On the other hand, however, the possibility still remains that oleate is required as a metabolite, and it may well be that it performs both metabolic and adsorptive functions. The effective concentration of oleate was about one-twentieth of that of albumin, which might be explained if its micelle is larger than the albumin molecule. When in the presence of albumin, oleate was required at four times the concentration, indicating the expected combination with albumin resulting in loss of activity. It is of interest to note that a loss of activity was also seen in the presence of polyvinyl alcohol.

Comparison of the medium described above with that of Talmadge & Herriott shows several differences. These authors used only seven amino acids but the constituents included biotin, choline chloride and inositol, which were not necessary for the strains used in the present work. Nucleosides, including thymidine, were used by

Talmadge & Herriott, whereas the free purines and pyrimidines were found sufficient in the medium described here and thymine was unnecessary. An interesting difference is that no added zinc ion was needed by the American authors; their medium also included triethanolamine, glycylglycine and Tween 80.

The defined medium described above for *Haemophilus* should now make it possible to study its metabolism and the genetic control of its metabolic processes.

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